

## Experimental Induction of Fluconazole Resistance in *Candida tropicalis* ATCC 750

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*Candida tropicalis* is less commonly isolated from clinical specimens than *Candida albicans*. Unlike *C. albicans*, which can be occasionally found as a commensal, *C. tropicalis* is almost always associated with the development of fungal infections. In addition, *C. tropicalis* has been reported to be resistant to fluconazole (FLC). To analyze the development of FLC resistance in *C. tropicalis*, an FLC-susceptible strain (ATCC 750) (MIC = 1.0 µg/ml) was cultured in liquid medium containing increasing FLC concentrations from 8.0 to 128 µg/ml. The strain developed variable degrees of FLC resistance which paralleled the concentrations of FLC used in the medium. The highest MICs of FLC were 16, 256, and 512 µg/ml for strains grown in medium with 8.0, 32, and 128 µg of FLC per ml, respectively. Development of resistance was rapid and could be observed already after a single subculture in azole-containing medium. The resistant strains were cross-resistant to itraconazole (MIC > 1.0 µg/ml) and terbinafine (MIC > 512 µg/ml) but not to amphotericin B. Isolates grown in FLC at concentrations of 8.0 and 32 µg/ml reverted to low MICs (1.0 µg/ml) after 12 and 11 passages in FLC-free medium, respectively. The MIC for one isolate grown in FLC (128 µg/ml) (128 R) reverted to 16 µg/ml but remained stable over 60 passages in FLC-free medium. Azole-resistant isolates revealed upregulation of two different multidrug efflux transporter genes: the major facilitators gene *MDR1* and the ATP-binding cassette transporter *CDR1*. The development of FLC resistance in vitro correlated well with the results obtained in an experimental model of disseminated candidiasis. While FLC given at 10 mg/kg of body weight/day was effective in reducing the fungal burden of mice infected with the parent strain, the same dosing regimen was ineffective in mice infected with strain 128 R. Finally, the acquisition of in vitro FLC resistance in strain 128 R was related to a loss of virulence. The results of our study elucidate important characteristics and potential mechanisms of FLC resistance in *C. tropicalis*.

The risk of opportunistic infections is greatly increased in patients who are severely immunocompromised due to cancer chemotherapy, organ or bone marrow transplantation, and to human immunodeficiency virus infection (37–39). Although *Candida albicans* is the organism most often associated with serious fungal infections, other *Candida* species have emerged as clinically important pathogens associated with opportunistic infections (5, 37–39). This is due to several reasons. First, a major effort in yeast species identification has been made in diagnostic laboratories. Second, the introduction of particular surgical devices, prostheses, and indwelling intravenous catheters has increased the risk of yeast infections due to *Candida* species which originate mainly from the environment (37, 38). Third, the widespread use of new antifungal molecules, especially fluconazole (FLC), has selected *Candida* species that are intrinsically resistant to this triazole, such as *Candida krusei*, or whose resistance is easily inducible, such as *Candida glabrata* (2, 10, 23, 26, 28, 37–39). Another non-*C. albicans* species of considerable clinical importance is *Candida tropicalis* (39). This species of *Candida* is less commonly isolated from clinical specimens than *C. albicans*. Unlike *C. albicans*, which can be occasionally found as a commensal, *C. tropicalis* is almost al-

ways associated with the development of fungal infections (39). In addition, *C. tropicalis* has been reported to be often resistant to FLC (14, 21).

So far, three mechanisms of azole-resistance have been described in *C. albicans* and *C. glabrata*: failure to accumulate drug intracellularly, increased production of the azole target enzyme, a lanosterol 14- $\alpha$ -demethylase called Erg11, and point mutations in the *ERG11* gene, the products of which have reduced affinity to azoles (1, 11, 12, 15–19, 29–36). The first mechanism of azole resistance may be caused by a lack of drug penetration due to change in membrane lipids or sterols or by active efflux of drugs resulting from upregulation of either *CDR* genes (encoding ABC transporters), effective against many azole drugs, or *MDR* (encoding major facilitators), specific for FLC (1, 11, 12, 15–19, 29–36). Few data are yet available on the mechanisms of azole resistance in *C. tropicalis* (9).

In this study, we developed an in vitro model to analyze the development of FLC resistance in *C. tropicalis*.

### MATERIALS AND METHODS

**Isolates.** *C. tropicalis* ATCC 750 was used throughout this study. *C. krusei* ATCC 6258 was used as control organism for experiments of antifungal susceptibility.

**Drugs.** Standard antifungal powders of FLC, itraconazole (ITC), terbinafine (TRB), and amphotericin B (AMB) were obtained from their respective manufacturers. Stock solutions were prepared in water (FLC), polyethylene glycol (ITC and TRB), and dimethyl sulfoxide (AMB). Antifungal agents were diluted with RPMI 1640 medium containing 2% glucose (RPMI 1640-G; Sigma Chemical, Milano, Italy) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid buffer (MOPS; Sigma).

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**Development of FLC resistance. (i) Strategy for induction of FLC resistance.** A single *C. tropicalis* colony was used to inoculate 10 ml of RPMI 1640-G which was incubated overnight in a rotating drum at 35°C. An aliquot of this culture containing  $10^6$  cells was transferred to 10 ml of medium containing 8.0, 32, or 128 µg of FLC per ml, and the cells were incubated as described above. When the cultures reached a density of approximately  $10^8$  cells/ml, aliquots containing  $10^8$  cells were transferred into fresh medium containing the same respective FLC concentration and reincubated. At each passage, a 1-ml aliquot of the suspension was mixed with 0.5 ml of 50% glycerol, and the mixture was frozen at -70°C for antifungal susceptibility testing as described below.

**(ii) Stability of FLC resistance in vitro.** Isolates found to exhibit FLC resistance were serially cultured in FLC-free medium. After each subculture, antifungal susceptibility testing was performed as described below. Passages were continued until the FLC MIC returned to the level of the parental strain.

**Antifungal susceptibility testing. (i) Procedure.** Susceptibility testing was performed exactly as outlined by the NCCLS method (20). Briefly, testing was performed in RPMI 1640-G buffered to pH 7.0 with MOPS. Two sets of FLC microtiter plates were prepared: in the first set the drug was tested at concentrations ranging from 0.125 to 64 µg/ml; in the other set the drug was tested at concentrations ranging from 1.0 to 512 µg/ml. ITC, TRB, and AMB were tested at concentrations ranging from 0.007 to 4.0 µg/ml, from 0.5 to 256 µg/ml, and from 0.03 to 16 µg/ml, respectively. Before reading, microtiter plates were sealed and then agitated for 5 min on a microtiter plate shaker. Readings were performed spectrophotometrically with an automatic plate reader (model MR 700; Dynatech) set at 490 nm. For both triazoles and TRB the MICs were defined as the first concentration of drug at which turbidity in the well was  $\leq 80\%$  of that in the control well. For AMB the MIC was defined as the first concentration of drug at which turbidity in the well was  $\leq 90\%$  of that in the control well (20).

**(ii) Definition.** According to the recent proposed breakpoints (20, 27), the isolates were defined as follows: FLC and ITC susceptible if the MICs were  $\leq 8.0$  µg/ml and  $\leq 0.125$  µg/ml, respectively; FLC and ITC susceptible-dose dependent if the MICs ranged from 16 to 32 µg/ml and from 0.25 to 0.5 µg/ml, respectively; or FLC and ITC resistant if the MICs were  $\geq 64$  and  $\geq 1.0$  µg/ml, respectively (20, 27).

**Phenotypic analysis of isolates.** The parent strain (ATCC 750) and one drug-resistant and revertant isolate from each drug exposure group were analyzed phenotypically.

**(i) Sugar assimilation.** The biochemical patterns of sugar assimilation were determined with the API 20C system (bioMérieux, Marcy l'Etoile, France) as specified by the manufacturer.

**(ii) Enzymatic analysis.** Suspensions of cells grown for 48 h in RPMI 1640-G or the cell supernatants were tested for enzymatic activity with the API ZYM system (bioMérieux) as specified by the manufacturer.

**(iii) Growth curves.** The growth rates were determined by incubating the isolates at 35°C in RPMI 1640-G with shaking. Absorbances were measured at 650 nm, and the cell doubling time was calculated for each isolate.

**Northern blot analysis.** The parent strain and selected FLC-resistant and FLC-susceptible isolates from each drug exposure group were analyzed for overexpression of FLC resistance genes. Briefly, total RNA from different isolates grown to mid-logarithmic phase in YEPD medium (1% yeast extract, 2% glucose, 2% peptone) was obtained by using the RNeasy mini kit (Qiagen Inc., Santa Clara, Calif.) following the manufacturer's instructions. RNA was separated by electrophoresis and subsequently transferred to Crenescrem Plus nylon membranes (Dupont NEN). *CtMDR1* (GenBank accession no. AF194419) was isolated from genomic DNA by PCR amplification using primers designed from the *C. albicans* *CaMDR1* gene. The primers used were BEN 36 (5' ACCCCA WGCHACWGGATADT 3') and BEN 56 (5' TTATWYGTTMTTGTTATG GTSTWGG 3'). Primer amplification was carried out with AmpliTaq (Perkin-Elmer) under the following conditions. A first cycle of denaturation for 4 min at 94°C which was followed by 30 cycles of annealing at 50°C for 2 min, elongation at 72°C for 2 min, and denaturation at 94°C for 30 s. A final elongation step at 72°C for 10 min completed the PCR. Hybridization with the *C. albicans* *CDR1* gene was performed with a  $^{32}$ P-labeled probe corresponding to the entire *CDR1* open reading frame. Low-stringency hybridization with the *CDR1* labeled probe was performed at 42°C in order to detect the *CDR1*-like mRNA in *C. tropicalis*. The low-stringency buffer consisting of 20% formamide, 1% sodium dodecyl sulfate (SDS), 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, and salmon sperm DNA (100 µg/ml). Other hybridizations were carried out under high-stringency conditions with the same buffer but containing 50% formamide. The *TEF3* gene from *C. albicans* was used as the RNA loading control as previously described (32). Probes were labeled by random priming (Amersham), and hybridizations were performed as described by Sanglard et al. (32). After hybridizations, blots were washed at 60°C as recommended by the manufacturer and subjected to autoradiography.

**Animal studies.** The parent strain ATCC 750 and the isolate grown in FLC (128 µg/ml) were tested in a murine model of disseminated candidiasis. Inbred BALB/c female mice weighing 23 to 26 grams were used throughout the study. One day before infection the organisms were inoculated into brain heart infusion broth and were incubated for 24 h at 35°C on a gyratory shaker (200 rpm). Organisms were harvested by low-speed centrifugation (1,500 × g), washed three times in sterile 0.9% saline, counted with a hemacytometer, and suspended in sterile saline to the desired concentrations. All studies were performed by chal-

TABLE 1. In vitro activities against *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258<sup>a</sup>

Isolate	MICs <sup>b</sup> (µg/ml) of:			
	FLC	ITC	TRB	AMB
<i>C. tropicalis</i>	1.0	0.125	32	0.5 (0.25–0.5)
<i>C. krusei</i>	32 (16–32)	0.5	32	0.5 (0.5–1.0)

<sup>a</sup> Each antifungal agent was tested from 9 to 29 times against both isolates.

<sup>b</sup> Values are medians (ranges shown in parentheses).

lenging the mice with an inoculum given in 0.2 ml of sterile saline administered in the lateral tail vein. Inoculum sizes were confirmed by quantitative cultures on Sabouraud dextrose agar (SDA) plates.

**(i) Virulence.** To assess the possible differences between isolates in levels of virulence, mice were infected with the same inoculum size of both the parent and the resistant strain. Mice were observed daily for 30 days, and survival was recorded. In other experiments, infected mice were sacrificed 5 and 30 days postinfection, both kidneys were excised by a sterile technique, weighed, and homogenized in 2.0 ml of sterile 0.9% saline. The homogenates were diluted by serial 10-fold dilution in sterile saline, and 0.1 ml of each dilution and the undiluted homogenate were cultured in triplicate on SDA. Culture plates were incubated for 48 h at 35°C, the numbers of CFU were then counted, and the number of CFU per gram of tissue was calculated.

**(ii) Stability of FLC resistance.** Mice infected with the FLC-resistant strain were sacrificed at predetermined time intervals, and both kidneys and the spleen were excised, homogenized, and plated onto SDA. After 48 h of incubation at 35°C, multiple colonies were selected from each plate and tested against FLC as described above.

**(iii) Azole treatment.** Mice infected with both the parent and the resistant strains were randomized into control and treatment groups, and FLC therapy was initiated at 24 h postchallenge. FLC was administered at 0.2 ml by intraperitoneal injection (25). Treatment was given once a day at 10 mg/kg of body weight and continued for 5 days. Control mice were given 0.2 ml of sterile saline. At 24 h after the administration of the last dose of FLC, the mice were sacrificed; both kidneys were excised, weighed, and homogenized; and the number of CFU per gram of tissue was calculated as described above.

**(iv) Statistical analysis.** Data from survival and organ clearance studies were analyzed by the Mann-Whitney U test, and significance was defined as  $P < 0.05$ .

## RESULTS

Table 1 shows FLC, ITC, TRB, and AMB MICs for *C. tropicalis* ATCC 750 and *C. krusei* ATCC 6258. Median FLC and ITC MICs for *C. tropicalis* ATCC 750 were 1.0 µg/ml and 0.125 µg/ml, respectively. According to NCCLS standards this isolate is susceptible to both triazoles (20, 27).

**Development of FLC resistance.** To analyze the development of FLC resistance, *C. tropicalis* ATCC 750 was cultured in medium containing FLC at concentrations of 8.0, 32, or 128 µg/ml. In general, isolates developed variable degrees of FLC resistance depending on the concentrations of FLC used in the medium (Fig. 1). The MICs for organisms grown in FLC at 8.0 µg/ml rose from 1.0 to 8.0 µg/ml after two passages, which was equivalent to 2 days of drug exposure. The highest MIC of FLC for this treatment group was 16 µg/ml, which was reached after 6 days of drug exposure. This value remained stable from day 8 to day 22 (Fig. 1A). Similarly, the MICs for organisms grown in FLC at 32 µg/ml rose rapidly, from 1.0 to 64 µg/ml after two passages, or 4 days of drug exposure. However, for this treatment group two further increases of FLC MIC were measured: from 64 to 128 µg/ml and from 128 to 256 µg/ml, after 21 and 26 days of drug exposure, respectively (Fig. 1B). The parent strain cultured directly in FLC at 128 µg/ml failed to reach an appropriate optical density, even after a prolonged incubation time. Therefore, the organisms grown in FLC at 32 µg/ml were passaged after 18 days of drug exposure (FLC MIC, 64 µg/ml) to FLC at 128 µg/ml (Fig. 1C). This strategy of induction of resistance resulted in an increase of FLC MIC from 64 to 256 µg/ml within 24 h. After 14 days of drug exposure at this concentration, the organism showed a further increase of FLC

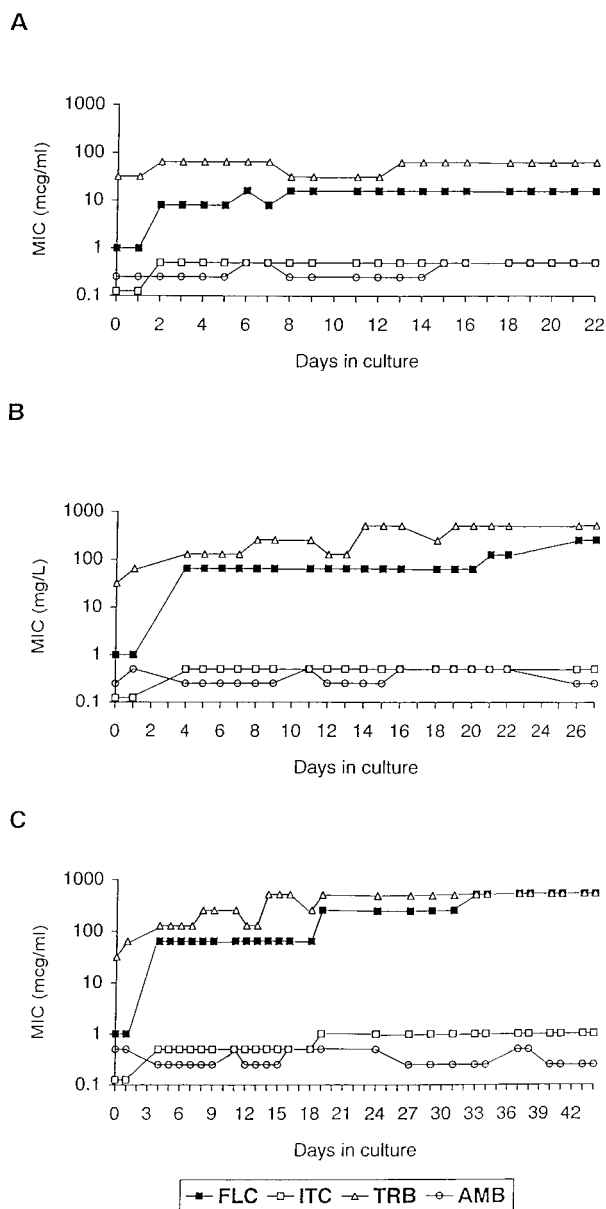


FIG. 1. Variations of FLC, ITC, TRB, and AMB MICs for isolates grown in medium containing 8.0 (A), 32 (B), and 128 (C) µg of FLC per ml. Each datum point represents one passage.

MIC (from 256 to 512 µg/ml) and was stable for up to 11 days in drug-containing medium (Fig. 1C).

**Susceptibilities to ITC, TRB, and AMB.** In order to measure the development of cross-resistance to other antifungal agents, we performed MIC assays with three antifungal agents with the organisms grown in separate FLC concentrations. The MICs of ITC for organisms cultured in FLC at both 8.0 and 32 µg/ml rose from 0.125 to 0.5 µg/ml after 2 and 4 days of FLC exposure, respectively. The increase of ITC MICs paralleled exactly the increase of FLC MIC (Fig. 1A and 1B). The MICs of ITC for organisms grown in FLC at 128 µg/ml increased rapidly to 1.0 µg/ml. However, even for this FLC treatment group, the ITC MIC did not increase further and remained stable for up to 44 days (Fig. 1C). The organisms grown in FLC at 8.0 µg/ml showed stable TRB MICs, ranging from 32 to 64 µg/ml (Fig.

1A). The MICs of TRB for the organisms grown in FLC at 32 µg/ml increased from 32 to 128 µg/ml and paralleled the increases of FLC and ITC MICs. The highest TRB MIC (>256 µg/ml) was reached after 14 days of FLC exposure and remained stable up to 31 days (Fig. 1B and 1C). AMB MICs for all organisms did not vary significantly and ranged from 0.25 to 0.5 µg/ml.

**Stability of azole resistance in vitro.** To assess the stability of FLC resistance, organisms grown in separate FLC concentrations were serially cultured in drug-free medium, and both FLC and ITC MICs were assayed after subcultures (Fig. 2). The isolates grown in FLC concentrations of 8.0 and 32 µg/ml reverted to the low MIC (1.0 µg/ml) after 12 and 11 days of growth in FLC-free medium, respectively (Fig. 2A and 2B). Although the MIC of FLC for the isolate grown in FLC at 128

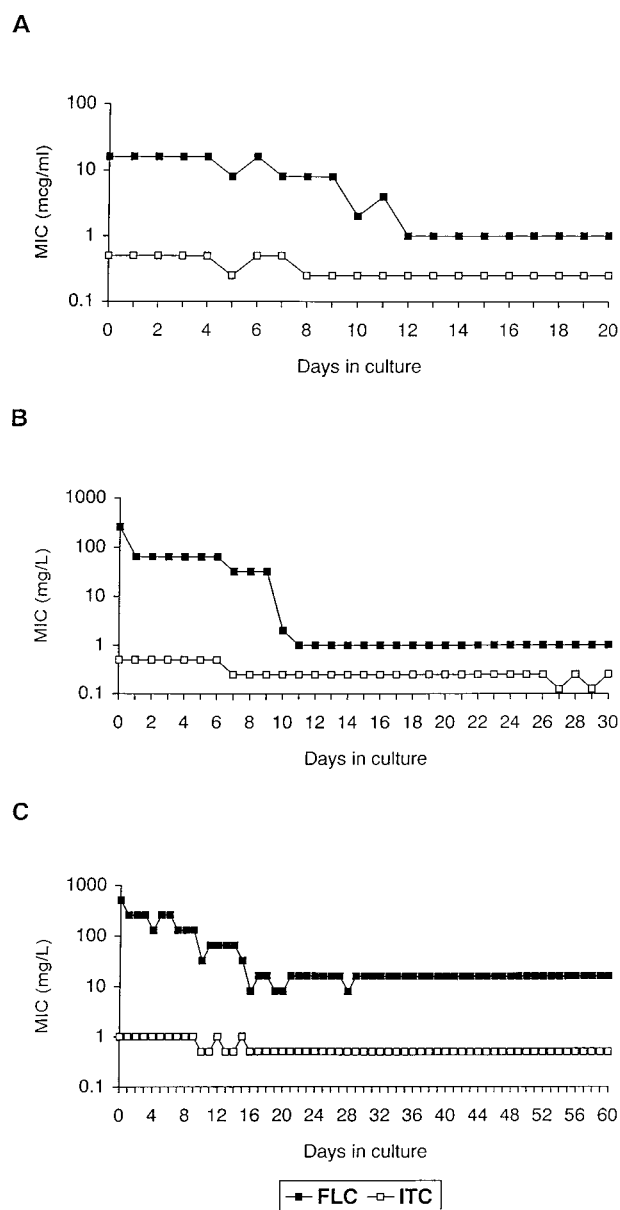


FIG. 2. Variations of FLC and ITC MICs for isolates previously grown in medium containing 8.0 (A), 32 (B), and 128 (C) µg of FLC per ml. Each datum point represents one passage.



TABLE 2. Growth rates of FLC-susceptible and -resistant phenotypes of *C. tropicalis* ATCC 750

Strain <sup>a</sup>	Doubling time (h)
Parent.....	2.2
8-R.....	3.7
8-S.....	2.2
32-R.....	3.8
32-S.....	2.3
128-R.....	4.5
128-S.....	2.5
128-mouse <sup>b</sup> .....	5.0

<sup>a</sup> The numbers indicate FLC concentrations (micrograms per milliliter) in medium; S, susceptible phenotype; R, resistant phenotype.

<sup>b</sup> This isolate, previously grown in FLC at 128 µg/ml, was recovered from the kidney of a mouse sacrificed 30 days postinfection (FLC MIC, 256 µg/ml).

µg/ml was significantly reduced (greater-than-fourfold dilutions) after 15 days of growth in FLC-free medium, the MIC of FLC for this isolate remained higher than that for the initial FLC-susceptible isolate up to 60 days (FLC MICs ranged from 8.0 to 16 µg/ml) (Fig. 2C). In general, ITC MICs decreased as FLU MICs decreased, and eventually all organisms reverted to the ITC-susceptible (ITC MIC, 0.125 µg/ml) or ITC-susceptible-dose-dependent (ITC MIC, 0.25 to 0.5 µg/ml) phenotypes. TRB MICs were determined for one organism in each FLC treatment group, and all reverted to the MIC observed for the parent strain (data not shown).

**Phenotypic analysis.** The patterns of sugar assimilation for the parent strain and both resistant and revertant isolates from each FLC treatment group were similar, and the same code (2556175) was obtained for all organisms tested. Although the enzymatic patterns of whole cells were quite stable among the strains, the resistant phenotypes revealed lower activities than those observed in the susceptible phenotypes for the following enzymes: alkaline phosphatase, caprylate esterase lipase, myristate lipase, two arylamidases (leucine and cystine), naphthol-AS-BI-phosphohydrolase, and α-glucosidase. Furthermore, no cystine-arylamidase activity was detected for the organism grown in FLC at 128 µg/ml, even upon recovering it from the kidneys of infected mice. Acid phosphatase was the only enzyme detected in the filtrates of culture supernatants of all strains, with the exception of the filtrates obtained from the organisms grown in FLC at 128 µg/ml (data not shown). Growth curves revealed that doubling times for FLC-resistant phenotypes were significantly longer than those for FLC-susceptible counterparts (Table 2).

**Expression of *CtMDR1* and *CDR1*.** Since multidrug efflux transporters are known to be involved in azole resistance in *C. albicans*, this possibility was explored with the strain used in the present study. Multidrug efflux transporters from two different families (ATP-binding cassette [ABC] transporters and major facilitators) were first cloned by PCR with primers matching the consensus sequences of both families. A *CDR*-like fragment and a *CaMDR1*-like fragment were recovered from *C. tropicalis*. From Northern analysis with RNA extracted from the strain used in the present study, only the *CaMDR1*-like gene (*CtMDR1*) showed expression patterns consistent with an involvement in FLC resistance. Representative results are reported in Fig. 3. The parent strain did not show any constitutive level of expression of *CtMDR1*. The FLC-resistant isolates grown in FLC at 8.0, 32, and 128 µg/ml all revealed overexpression of *CtMDR1*, while in their respective revertant isolates gene expression was reduced to background levels. *CtMDR1* was also upregulated in the strain grown in FLC at

128 µg/ml which was recovered from the kidneys of untreated mice 30 days postinfection (Fig. 3). However, upregulation of *MDR1* genes from *Candida* species only confers resistance to FLC (30, 32). Since cross-resistance to other azole derivatives was observed in the FLC-resistant isolates of the present study, the single overexpression of *CtMDR1* could not account for this feature. It was still possible that multidrug transporters of the ABC superfamily, which take as substrates different azole derivatives, could still be upregulated in the *C. tropicalis* azole-resistant isolates. Therefore, in order to evaluate this hypothesis, we performed low-stringency hybridizations with the entire open reading frame of the ABC transporter gene *CDR1* from *C. albicans*. As shown in Fig. 3, the signal corresponding to the mRNA matching *CDR1* increased in parallel to those of *CtMDR1*. These results strongly suggest that a *C. tropicalis* *CDR1* gene homologous to *CDR1* is upregulated in isolates with increasing FLC MICs. Thus, azole cross-resistance observed in the *C. tropicalis* isolates of the present study could be explained by the simultaneous upregulation of both multidrug transporters of two different families.

**Animal experiments.** To assess the virulence of *C. tropicalis* ATCC 750, we performed initial experiments by challenging the mice (10 mice per group) with a broad range of inoculum sizes, i.e.,  $5 \times 10^4$ ,  $5 \times 10^5$ , and  $5 \times 10^6$  CFU per mouse. Only in the third group we observed a mortality rate >50% within 30 days. In addition, while the first two groups of animals cleared the infection within few days, mice infected with the larger inoculum showed a fungal density of log 3 CFU/g of kidneys on day 30 after the challenge (data not shown). Therefore, further experiments were performed with an inoculum of  $10^7$  CFU per mouse. Experiments were performed with the parent strain and the strain grown in FLC at 128 µg/ml. There was a dramatic difference in the survival rate between animals injected with the parent strain (60% mortality) and those challenged with the resistant phenotype (0% mortality;  $P = 0.0001$ ) (Fig. 4A). Mouse survival was mirrored in the kidney fungal burdens of infected mice (Fig. 4B). The fungal burdens in animals infected with the parent strain resulted in significantly higher counts than those observed in animals infected with the resistant phenotype either 5 ( $P = 0.019$ ) or 30 days ( $P = 0.0001$ ) postinfection.

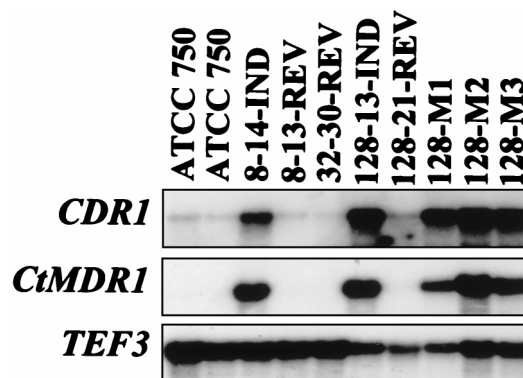


FIG. 3. Variation of *CtMDR1* and *CDR1* expression in FLC-susceptible and -resistant phenotypes of *C. tropicalis* ATCC 750. Lanes: ATCC 750, parent strain; 8-14-IND, isolate cultured in FLC at 8.0 µg/ml (14th passage); 8-13-REV, isolate previously cultured in FLC at 8.0 µg/ml and then passaged in FLC-free medium (13th passage); 32-30-REV, isolate previously cultured in FLC at 32 µg/ml and then passaged in FLC-free medium (30th passage); 128-13-IND, isolate cultured in FLC at 128 µg/ml (13th passage); 128-21-REV, isolate previously cultured in FLC at 128 µg/ml and then passaged in FLC-free medium (21st passage); 128-M1, 128-M2, and 128-M3, isolates cultured in FLC at 128 µg/ml and recovered from the kidneys of three mice 30 days postinfection.

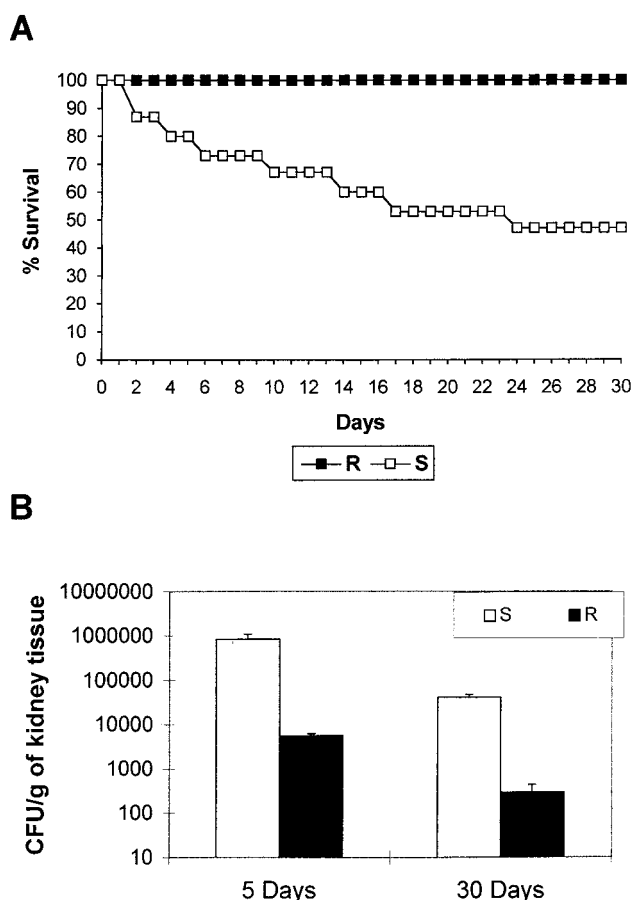


FIG. 4. Virulence of FLC-susceptible (S [parent strain]; FLC MIC, 1.0  $\mu$ g/ml) and FLC-resistant (R; organism cultured in FLC at 128  $\mu$ g/ml; FLC MIC, 256  $\mu$ g/ml) phenotypes of *C. tropicalis* ATCC 750 in an immunocompetent mouse model. Mice were inoculated with  $10^7$  CFU via the lateral tail vein. (A) Survival curves. There were 15 mice per group (B) CFU of strain ATCC 750-infected kidneys recovered from animals sacrificed on days 5 and 30 postinfection. There were four to seven mice per time interval or group. Error bars show standard deviations from the mean.

Further experiments were carried out to address the stability of FLC resistance in vivo with the strain grown in FLC at 128  $\mu$ g/ml. Groups of two to three mice each were sacrificed on days 5, 10, and 30 postinfection, and two to five random isolates per mouse recovered from both kidneys and spleens were tested for FLC susceptibility. All organisms retained the FLC-resistant phenotype over the test period (data not shown).

Table 3 shows fungal burdens of FLC treated mice. While FLC at 10 mg/kg of body weight was effective in reducing the number of CFU per gram of kidneys in mice infected with the parent strain ( $P < 0.05$ ), the same dosing regimen was ineffective in mice infected with the resistant phenotype.

## DISCUSSION

Mechanisms of azole resistance have been extensively investigated in strains of *C. albicans* isolated from patients with AIDS and oropharyngeal candidiasis (11, 16, 17, 29, 32, 34, 35). Recently, several studies elucidated the mechanisms of azole resistance in *C. glabrata* and *C. krusei* (12, 18, 22, 23). Little is known about azole resistance in *C. tropicalis* (9). This species has been reported as the second-most-frequent cause of candidemia in both neutropenic and nonneutropenic patients. Additionally, in contrast to *C. albicans*, which can be occa-

sionally found as a commensal, *C. tropicalis*, when encountered, is almost always associated with diseases (14, 39). It has been reported that *C. tropicalis* azole susceptibility can be reduced by drug exposure in vitro or in vivo (2, 14, 39). The dynamics of development of FLC resistance in this *Candida* species is largely unknown.

The results of our study underline several characteristics of the in vitro acquisition of FLC resistance in *C. tropicalis*. We showed here that the development of FLC resistance occurs very rapidly and that the degree of resistance is strongly related to the drug concentration utilized in the growth medium. In particular, the MIC of FLC for the strain grown in FLC at 32  $\mu$ g/ml increased from 1.0 to 64  $\mu$ g/ml after 4 days of drug exposure. Similarly, the strain previously grown in FLC at 32  $\mu$ g/ml and then passaged in FLC at 128  $\mu$ g/ml developed a very high degree of resistance within 24 h: from 64 to 256  $\mu$ g/ml. In addition, when the resistant strain grown in FLC at 32  $\mu$ g/ml was passaged in FLC-free medium, the MICs for this strain reverted to low values comparable to the one measured in the initial strain (12 passages in FLC-free medium). On the other hand, the strain grown in FLC at 128  $\mu$ g/ml retained a level of resistance (MIC, 16  $\mu$ g/ml) that was higher than that measured in the parent strain up to 60 days of passages in FLC-free medium. This finding correlated well with the animal experiment results. Following challenge with the strain grown in FLC at 128  $\mu$ g/ml, the yeasts recovered 30 days postinfection from the spleen or kidneys of untreated mice retained a high degree of FLC resistance.

All together these data indicate that the dynamics of development of FLC-resistance in this *Candida* sp. is quite different from that experimentally observed in *C. albicans* (8). For the latter species, the time required to develop FLC resistance as well as the time required to revert to baseline is longer. Calvet et al. (8) showed that even when the strain used in their study was originally passaged in FLC at 128  $\mu$ g/ml it acquired a very high degree of FLC resistance. Contrarily to these authors, we failed to obtain an appropriate optical density for cells grown in FLC at 128  $\mu$ g/ml, thus indicating that this drug concentration is very toxic to susceptible strains of *C. tropicalis*. In order to see whether the development of FLC resistance in *C. tropicalis* was characterized by a cross-resistance to other antifungals, we determined ITC, TRB, and AMB MICs for all the organisms grown in each FLC concentration. As repeatedly observed in *C. albicans*, ITC and TRB showed a progressive

TABLE 3. Fungal burden in kidney tissues of mice infected with *C. tropicalis* ATCC 750 and treated with FLC<sup>a</sup>

Isolate <sup>b</sup>	FLC dosage (mg/kg)	Median counts $\pm$ SE (CFU/g [ $10^4$ ])
S	0	211 $\pm$ 98.2
	10	31 <sup>c</sup> $\pm$ 3.1
R	0	12 $\pm$ 5.1
	10	6.5 <sup>d</sup> $\pm$ 1.9

<sup>a</sup> There were seven mice in each treatment group. The mice were randomized into control and treatment groups, and FLC therapy was initiated at 24 h postchallenge. FLC was administered at 0.2 ml by intraperitoneal injection. Treatment was given once a day at 10 mg/kg of body weight and continued for 5 days. At 24 h after the administration of the last dose of FLC, the mice were sacrificed.

<sup>b</sup> S, *C. tropicalis* ATCC 750 FLC susceptible (FLC MIC, 1.0  $\mu$ g/ml) (parent strain); R, *C. tropicalis* ATCC 750 FLC resistant (cultured in FLC at 128  $\mu$ g/ml; FLC MIC, 256  $\mu$ g/ml).

<sup>c</sup> Treatment reduced the count significantly below that in the tissues of controls ( $P < 0.05$ ).

<sup>d</sup> Treatment did not reduce the count significantly below that in the tissues of controls ( $P = 0.323$ ).

increase in their respective MICs which paralleled exactly the increase of FLC MICs (3, 17, 24, 29, 31, 34). As expected, AMB MICs remained stable over time.

The increase in FLC and ITC MICs was correlated with increases of both *CtMDR1* and *CDR1* expression. Because we did not clone a *CDR1*-like homologue from *C. tropicalis*, we assumed in this study that the *C. albicans* *CDR1* probe would reveal the *C. tropicalis* homologous *CDR1* gene in low-stringency hybridizations. As shown in Fig. 3, this was effectively the case. Strains for which the MICs of FLC reverted to low values did not express *CtMDR1* at detectable levels. However, in these strains, basal *CDR1* expression was still detected, as reported in most azole-susceptible *C. albicans* isolates (1, 11, 16, 17, 19). Interestingly, the resistant phenotype recovered 30 days postinfection from the kidneys of untreated mice showed the maintenance of the upregulation of both genes. Without excluding the possibility that other azole resistance mechanisms could operate in azole-resistant isolates of this study, these data show that, as seen in *C. albicans*, one of the possible mechanisms involved in FLC resistance is the active efflux of drug due to an upregulation of multidrug efflux transporter genes (1, 11, 16, 17, 31, 32, 34). Simultaneous upregulation of both types of transporters by in vitro exposure to FLC has not been yet described in other yeast species. Albertson et al. (1) obtained a FLC-resistant strain in *C. albicans* overexpressing *CaMDR1*; Marr et al. (19) were also able to transiently downregulate *CDR1* following in vitro passages of azole-resistant *C. albicans* isolates in drug-free medium.

Although the resistant phenotype did not respond to 10 mg of FLC/kg/day in a mouse model of systemic candidiasis, this strain was dramatically less virulent than the parent strain as shown by the lack of mortality and the lower number of CFU/gram of infected tissues. The relationship between virulence and antifungal resistance is controversial, and the limited data available concern mainly *C. albicans* strains (4, 6, 8, 13). In an earlier study of two pairs of serial isolates of *C. albicans* with identical DNA patterns obtained from two patients with first responsive and then refractory thrush, we found that pre- and posttreatment isolates from both patients were equally virulent in an animal model of murine candidemia (4). Recently, Graybill et al. (13) showed that decreased virulence of serial *C. albicans* isolates is associated with increasing FLC MICs in some but not all cases. Interestingly, these authors found that the isolates for which the MICs of FLC were high which had low virulence were associated with FLC failure when they were tested in mice with candidal infections. In contrast, the in vitro resistant isolates which had high virulence, responded to FLC therapy with standard doses in the same animal model (13). The reduced virulence of the *C. tropicalis* resistant strain compared with that of the parent strain found in this study could be due to the experimental acquisition of FLC resistance. The phenotypic characteristics of the resistant strains were profoundly altered compared with those of the parent and the revertant strains. These data would indicate a reduced fitness of the resistant phenotype and would explain its reduced capability to establish an infection. Our data agree with those recently observed by Buckner et al. (7) in *Trypanosoma cruzi*. They found that the azole-resistant mutant was less virulent than the parent strain.

In summary, we evaluated an in vitro model to analyze the development of FLC resistance in *C. tropicalis*. In this model the acquisition of azole resistance occurs very rapidly. Multidrug transporter genes of two different families, the ABC transporters and the major facilitators, are upregulated in the resistant phenotypes, indicating that one of the mechanisms of resistance in this *Candida* sp. may be drug efflux from the cell.

Similar to observations by others in several eukaryotes, the resistant phenotype appears less virulent than the parent strain.

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